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Real-Time Nucleic Acid Sequence-Based Amplification Is More Convenient than Real-Time PCR for Quantification of *Plasmodium falciparum*

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Determination of the number of malaria parasites by routine or even expert microscopy is not always sufficiently sensitive for detailed quantitative studies on the population dynamics of *Plasmodium falciparum*, such as intervention or vaccine trials. To circumvent this problem, two more sensitive assays, real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) and real-time quantitative PCR (QT-PCR) were compared for quantification of *P. falciparum* parasites. QT-NASBA was adapted to molecular beacon real-time detection technology, which enables a reduction of the time of analysis and of contamination risk while retaining the specificity and sensitivity of the original assay. Both QT-NASBA and QT-PCR have a sensitivity of 20 parasites/ml of blood, but QT-PCR requires a complicated DNA extraction procedure and the use of 500 μl of venous blood to achieve this sensitivity, compared to 50 μl of finger prick blood for real-time QT-NASBA. Both techniques show a significant correlation to microscopic parasite counts, and the quantification results of the two real-time assays are significantly correlated for in vitro as well as in vivo samples. However, in comparison to real-time QT-PCR, the results of real-time QT-NASBA can be obtained 12 h earlier, with relatively easy RNA extraction and use of finger prick blood samples. The prospective development of multiplex QT-NASBA for detection of various *P. falciparum* developmental stages increases the value of QT-NASBA for malaria studies. Therefore, for studies requiring sensitive and accurate detection of *P. falciparum* parasites in large numbers of samples, the use of real-time QT-NASBA is preferred over that of real-time QT-PCR.

Routine clinical diagnosis of malaria is usually based on microscopic detection of *Plasmodium* parasites in blood smears. However, this technique is relatively laborious when large numbers of samples need to be quantified simultaneously. Furthermore, the detection limit of microscopy, 1 to 20 parasites per μl of blood, may not always be sufficiently sensitive. Parasite densities below the detection level of microscopy may play an important role in *Plasmodium* population dynamics and the epidemiology of the disease; therefore, the availability and use of more-sensitive detection techniques is a prerequisite for many research projects. With the rapid developments in the field of molecular biology, several nucleic acid-based amplification methods, including PCR, reverse transcriptase PCR, and nucleic acid sequence-based amplification (NASBA) (2, 3, 7–9, 11), are now available for detection of *Plasmodium* parasites. Because many studies require accurate and sensitive quantification of parasites, most of these techniques have been adapted for quantitative analysis (1, 3, 6, 7, 11). In preparation for vaccine trials and epidemiological studies directed at evaluation of interventions, the present study was designed to evaluate the newly developed real-time quantitative NASBA (QT-NASBA) and to compare the assay to another quantitative nucleic acid-based method used for quantification of *P. falciparum* parasites, real-time quantitative PCR (QT-PCR).

Schoone et al. (7) developed a QT-NASBA based on the detection of *Plasmodium falciparum* 18S rRNA with a sensitivity of 10 to 50 parasites/ml (7). This method can be applied to 50-μl finger prick blood samples and has complete specificity for *P. falciparum*. This QT-NASBA assay is a combination of RNA extraction, amplification of the RNA target plus an internal control, and end point detection of amplification products by electrochemiluminescence (ECL). Competitive coamplification of an internal control enables accurate quantification (5, 7). In the present study, the QT-NASBA was adapted to real-time molecular beacon technology (10) to avoid inaccurate quantification at high parasite densities, which may occur with end point detection due to depletion of reagents during the amplification reaction. Moreover, the closed-tube format of the assay greatly reduces the risk of contamination and thus of false-positive results. The real-time QT-NASBA allows accurate quantification of as many as 48 samples in 4 h, including RNA extraction.

The adaptation of the QT-NASBA to real-time quantification of *P. falciparum* parasites now allows direct comparison to real-time *P. falciparum* QT-PCR (3), which is based on detection of DNA encoding 18S rRNA. This real-time QT-PCR has a sensitivity of 20 parasites/ml of blood when 500-μl blood samples are used, with accurate quantification of 48 samples within 16 h.

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MATERIALS AND METHODS

Real-time QT-NASBA. Real-time *P. falciparum* QT-NASBA for 18S rRNA (GenBank accession number M19172.1) was performed on a NucliSens EasyQ analyzer (bioMérieux) using the Nuclisens Basic kit for amplification according to the manufacturer's manual at a KCl concentration of 80 mM. Reactions were carried out in a total volume of 10 μl per reaction. The forward primer was 5′-GTCATCTTTCGAGGTGACTT-3′ (nucleotides 1136 to 1155); the reverse primer was 5′-AATTCTAATACGACTCACTATAGGGAGAAGGAACTTTC TCGCTTGCGCGAA-3′ (T7 promoter sequence, linker, and nucleotides 1216 to 1235); the Pf18S molecular beacon was 5′–6-carboxyfluorescein–CGATCGG AGAAATCAAAGTCTTTGGG-CGATCG-dimethylaminoazosulfonic acid-3′ (molecular beacon stem of 6 paired nucleotides and nucleotides 1182 to 1201). The time to positivity, i.e., the time point during amplification at which the number of target amplicons detected became higher than the mean for three negative controls plus 20 standard deviations, was calculated. The use of a standard ring stage parasite dilution series allows exact calculation of the number of parasites present in unknown samples.

Real-time QT-PCR. Real-time QT-PCR analysis and subsequent calculations were performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems) using a fluorescently labeled TaqMan probe to enable continuous monitoring of amplicon formation. The procedures, primer, and probe sequences are those presented by Hermsen et al. (3), except that the probe is now labeled with tetrachloro-6-carboxyfluorescein (TET). A minimum of 0.5 ml of whole blood is needed to obtain a sensitivity of 20 parasites/ml of blood, and the blood needs to be filtered through a Plasmodipur filter to remove white blood cells that may cause viscosity problems during DNA extraction. The number of parasites is calculated from the threshold cycle, i.e., the amplification cycle number at which emitted fluorescence exceeds the baseline emission + 10 standard deviations.

In vitro samples. The detection limit and accuracy of quantification by real-time QT-NASBA were evaluated using purified ring stage parasites and purified stage IV to V gametocytes from in vitro culture of *P. falciparum* isolate NF54 (4). Purified parasites were diluted in whole blood to obtain 10-fold dilution series, ranging from 10 to 10^6 ring stage parasites ($n = 3$ for each concentration) and 10^2 to 10^7 gametocytes ($n = 4$ for each concentration) per ml of blood as counted by microscopy. RNA was independently extracted for each dilution series, and quantification by real-time QT-NASBA was performed three times for each ring stage sample and four times for each gametocyte sample.

For comparison with real-time QT-NASBA, the dilution series of 10 to 10^6 ring stage *P. falciparum* NF54 parasites per ml of blood was also used for DNA extraction and parasite quantification by real-time QT-PCR. Quantification by real-time QT-PCR was performed three times in duplicate.

In vivo samples. For direct comparison of samples from in vivo infections, 13 samples from an individual participating in a human experimental *P. falciparum* infection study were analyzed in duplicate both by real-time QT-NASBA and by real-time QT-PCR. The human experimental infection study, performed in preparation for vaccine trials, was approved by the ethics committee of the University Medical Centre Nijmegen (CWOM 0011–0262, 2002/170) and a detailed description of the study is given by Hermsen et al. (3). Real-time QT-PCR was used for early detection of parasites (2 to 4 days before microscopic detection of parasites and treatment [3]) in the volunteers, and real-time QT-NASBA was included for comparison, with the aim of evaluating QT-NASBA for future studies. Parasite quantification of in vivo samples by the two different assays was compared using Spearman's correlation test.

RESULTS

In vitro samples. Results of quantification of *P. falciparum* parasites by real-time QT-NASBA and real-time QT-PCR were compared with microscopic parasite counts. Highly significant correlations were found for quantification of ring stage parasites and gametocytes by NASBA (Fig. 1a) (for ring stages, $R^2 = 0.942$ and $P < 0.01$; for gametocytes, $R^2 = 0.966$ and $P < 0.01$) and for quantification of ring stage parasites by PCR (Fig. 1b) ($R^2 = 0.985$; $P < 0.01$).

The interassay variation of both assays was calculated as standard deviation/average × 100% (Table 1). The interassay variation includes variation of both amplification and nucleic acid extraction, because all samples were processed individu-

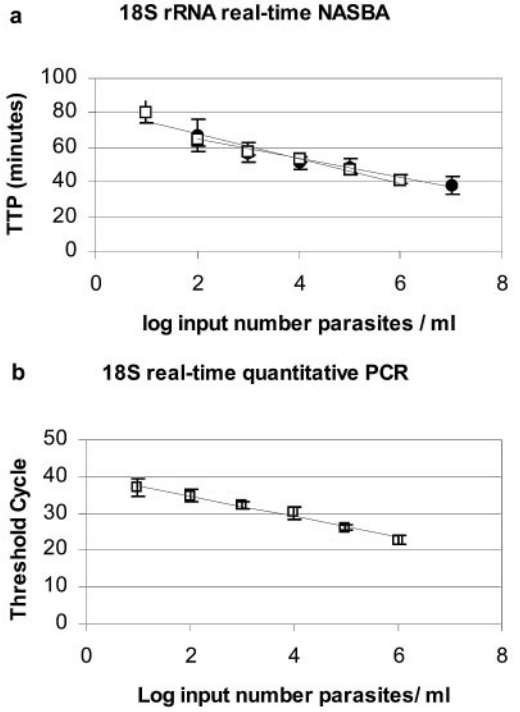

FIG. 1. (a) Mean number of *P. falciparum* ring stage parasites (circles) and gametocytes (squares) in a 10-fold dilution series quantified by real-time 18S rRNA QT-NASBA. Error bars, standard deviations. The log number of parasites per sample as counted by microscopy shows a highly significant correlation with the time to positivity (TTP) as calculated by real-time QT-NASBA (for ring stage parasites, $R^2 = 0.942$ and $P < 0.01$; for gametocytes, $R^2 = 0.966$ and $P < 0.01$). (b) Mean number of *P. falciparum* ring stage parasites in a 10-fold dilution series quantified by 18S real-time QT-PCR. The mean is calculated for 7 observations with 10 parasites/ml, 12 observations with 10^2 parasites/ml, and 18 observations with 10^3 to 10^6 parasites/ml. Error bars, standard deviations. The log number of parasites per sample as counted by microscopy shows a highly significant correlation with the threshold cycle as calculated by real-time QT-PCR ($R^2 = 0.985$; $P < 0.01$).

TABLE 1. Comparison of interassay variation for real-time QT-NASBA and real-time QT-PCR[a]

Assay and log input no./ml	Avg interassay variation (range)	Assay and log input no./ml	Avg interassay variation (range)
Real-time QT-NASBA		Real-time QT-PCR	
6	4.67 (3.62–6.16)	6	4.51 (1.86–8.75)
5	4.04 (1.09–6.81)	5	3.07 (0.81–6.42)
4	3.39 (2.68–4.29)	4	4.54 (1.17–12.65)
3	4.45 (3.19–6.60)	3	2.08 (1.13–4.13)
2	7.95 (6.01–9.78)	2	3.87 (1.65–8.75[b])
1	9.71 (7.48–12.41)	1	8.53 (4.84–10.84[c])

[a] Interassay variation for ring stage parasite quantification, calculated as standard deviation/mean × 100% for each parasite concentration. The average and range were calculated over three analyses on different days for real-time QT-NASBA and over three duplicate analyses on different days for real-time QT-PCR.
[b] $n = 4$.
[c] $n = 3$.

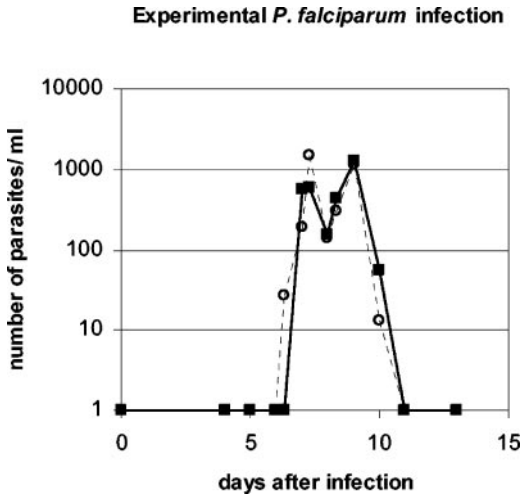

FIG. 2. Quantification of parasites in blood samples taken from a volunteer during a human experimental *P. falciparum* infection. Mean results from duplicate analyses are shown for real-time QT-NASBA (open circles) and real-time QT-PCR (solid squares). The results of quantification by the two techniques were significantly correlated (Spearman's $\rho = 0.930$; $P < 0.01$). Drug treatment was given at day 8.3 after infection, when parasites were detected by microscopy.

ally. For real-time QT-NASBA, the interassay variation tends to be low and relatively stable over the range of parasite concentrations tested, although the interassay variation seems to increase at the lowest tested parasite concentrations of 10 asexual parasites per ml of blood. The interassay variation of real-time QT-PCR is similar to that of real-time QT-NASBA but could not be calculated accurately for the lower concentrations due to negative results in the real-time QT-PCR (Table 1). The detection limit for both real-time QT-NASBA and real-time QT-PCR is 20 parasites/ml of blood.

In vivo samples. Parasite quantification results for the 13 samples taken during the course of an experimental *P. falciparum* infection are shown in Fig. 2 for real-time QT-NASBA and real-time QT-PCR. There was a significant correlation between the parasite quantification results by real-time QT-NASBA and those by real-time QT-PCR (Spearman's $\rho = 0.930$; $P < 0.01$).

DISCUSSION

The real-time QT-NASBA quantifies *P. falciparum* parasites with complete specificity and sensitivity, as was previously observed with the ECL-based QT-NASBA (7). However, the advantages of real-time QT-NASBA are that quantification results are obtained more rapidly and quantification of high parasite densities is not adversely affected by possible depletion of reagents in the amplification process, due to real-time detection of amplicons. Furthermore, results are obtained with less manual handling of samples than the ECL-based QT-NASBA requires, and the risk of carryover contamination and of amplicons spreading in the laboratory is greatly reduced due to the closed-tube format.

Comparison of real-time QT-NASBA and real-time QT-PCR for quantification of parasites in both control series from in vitro culture and in vivo samples from the human experimental *P. falciparum* infection study shows that the techniques correlate well. Both real-time QT-NASBA and real-time QT-PCR have a detection limit of 20 parasites/ml of blood. For real-time QT-NASBA, parasite densities of 10/ml can also be detected, but the detection limit is set to 20 parasites per ml of blood, i.e., 1 parasite per blood sample when 50-μl blood samples are used. For real-time QT-PCR, detection of 10 parasites/ml of blood is feasible but increases the risk of false-negative results, as shown in Fig. 1b and Table 1.

When the volumes of blood used for nucleic acid extraction and the amount of extract used in both real-time assays are taken into account, the sensitivity of real-time QT-NASBA is higher than that of real-time QT-PCR, with absolute detection limits of 0.05 and 1 parasite per reaction in QT-NASBA and QT-PCR, respectively. The higher sensitivity of the real-time QT-NASBA is attributed to the abundance of rRNA compared to that of 18S rRNA, the target of real-time QT-PCR.

Although the detection limit of the real-time QT-PCR is 20 parasites/ml of blood (3), in one of the parasite dilution series a concentration of 100 parasites/ml of blood repeatedly could not be detected by real-time QT-PCR (Fig. 1b and Table 1). Five independent standard parasite dilution series ranging from 5 to 10^6 parasites/ml of blood, with a total of 58 measurements, gave correlations with microscopic parasite counts similar to those presented in Fig. 1b. In these standard dilution series, both 100 parasites ($n = 5$) and 50 parasites ($n = 5$)/ml of blood were consistently detected. Considering the consistent results of standard dilution series, the negative results for detection of a parasite concentration of 100/ml of blood are probably due to a mistake made during the DNA extraction. Just below the detection limit, a concentration of 10 parasites/ml of blood cannot be detected in half of the samples.

In the present real-time QT-NASBA, 48 samples can be quantified within 4 h, including RNA extraction, compared to 16 h when real-time QT-PCR is used. Another advantage of QT-NASBA is the ability to use small sample volumes for analysis, enabling the use of finger prick blood samples as opposed to venous blood collection and mandatory filtering of blood samples for real-time QT-PCR. A recently adapted storage protocol eliminates the need to store RNA at −70°C or in liquid nitrogen, making the collection of RNA samples during field studies more convenient (P. Schneider et al., unpublished data). The specificity of QT-NASBA for RNA, without the need for complicated RNA extraction to remove all genomic DNA from the samples, enables further development of the assay for detection of various life stages of *P. falciparum* (6). The modification of ECL-based QT-NASBA to real-time QT-NASBA also allows multiplexing, i.e., the detection of multiple targets within one reaction. Real-time QT-NASBA will be a valuable tool for quantification of different developmental stages of *P. falciparum* in studies that e.g., evaluate the effect of control measures or investigate parasite population dynamics, epidemiology, or the transmission of the disease.

In conclusion, both the real-time QT-NASBA and real-time QT-PCR are reliable methods for quantification of *P. falciparum* parasites, with low risks of carryover contamination and amplicon contamination of laboratory working space. However, in comparison to real-time QT-PCR, the results of real-time QT-NASBA can be obtained 12 h earlier, with a relatively easy RNA extraction and the use of finger prick blood samples

instead of venous blood collection. The development of QT-NASBA for detection of various *P. falciparum* developmental stages (6) and its prospective development into a multiplex assay further increase the value of QT-NASBA for malaria studies. Therefore, the use of real-time QT-NASBA is preferred over that of real-time QT-PCR, especially in studies where large numbers of samples need to be quantified at higher levels of sensitivity than microscopic detection can provide, such as epidemiological surveys, drug efficacy trials, and vaccine trials.

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REFERENCES

1. **Bell, A. S., and L. C. Ranford Cartwright.** 2002. Real-time quantitative PCR in parasitology. Trends Parasitol. **18:**337–342.
2. **Ciceron, L., G. Jaureguiberry, F. Gay, and M. Danis.** 1999. Development of a *Plasmodium* PCR for monitoring the efficacy of antimalarial treatment. J. Clin. Microbiol. **37:**35–38.
3. **Hermsen, C. C., D. S. Telgt, E. H. Linders, L. A. van de Locht, W. M. Eling, E. J. Mensink, and R. W. Sauerwein.** 2001. Detection of *Plasmodium falciparum* malaria parasites in vivo by real-time quantitative PCR. Mol. Biochem. Parasitol. **118:**247–251.
4. **Ponnudurai, T., A. H. Lensen, J. F. Meis, and J. H. Meuwissen.** 1986. Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. Parasitology **93:**263–274.
5. **Schallig, H. D., G. J. Schoone, E. J. Lommerse, C. C. Kroon, P. J. de Vries, and T. van Gool.** 2003. Usefulness of quantitative nucleic acid sequence-based amplification for diagnosis of malaria in an academic hospital setting. Eur. J. Clin. Microbiol. Infect. Dis. **22:**555–557.
6. **Schneider, P., G. Schoone, H. Schallig, D. Verhage, D. Telgt, W. Eling, and R. Sauerwein.** 2004. Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. Mol. Biochem. Parasitol. **137:**35–41.
7. **Schoone, G. J., L. Oskam, N. C. M. Kroon, H. D. F. H. Schallig, and S. A. Omar.** 2000. Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. J. Clin. Microbiol. **38:**4072–4075.
8. **Smits, H. L., G. C. Gussenhoven, W. Terpstra, R. A. F. Schukkink, B. van Gemen, and T. van Gool.** 1997. Detection, identification and semi-quantification of malaria parasites by NASBA amplification of small subunit ribosomal RNA sequences. J. Microbiol. Methods **28:**65–75.
9. **Snounou, G., and H. P. Beck.** 1998. The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol. Today **14:**462–467.
10. **Tyagi, S., and F. R. Kramer.** 1996. Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. **14:**303–308.
11. **Witney, A. A., D. L. Doolan, R. M. Anthony, W. R. Weiss, S. L. Hoffman, and D. J. Carucci.** 2001. Determining liver stage parasite burden by real time quantitative PCR as a method for evaluating pre-erythrocytic malaria vaccine efficacy. Mol. Biochem. Parasitol. **118:**233–245.